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AIM AND SCOPE

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REVIEW

Detecting circulating tumor material and digital pathology imaging during pancreatic cancer progression

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Abstract

Pancreatic cancer (PC) is a leading cause of cancer-related death worldwide. Clinical symptoms typically present late when treatment options are limited and survival expectancy is very short. Metastatic mutations are heterogeneous and can accumulate up to twenty years before PC diagnosis. Given such genetic diversity, detecting and managing the complex states of disease progression may be limited to imaging modalities and markers present in circulation. Recent developments in digital pathology imaging show potential for early PC detection, making a differential diagnosis, and predicting treatment sensitivity leading to long-term survival in advanced stage patients. Despite large research efforts, the only serum marker currently approved for clinical use is CA 19-9. Utility of CA 19-9 has been shown to improve when it is used in combination with PC-specific markers. Efforts are being made to develop early-screening assays that can detect tumor-derived material, present in circulation, before metastasis takes a significant course. Detection of markers that identify circulating tumor cells and tumor-derived extracellular vesicles (EVs) in biofluid samples offers a promising non-invasive method for this purpose. Circulating tumor cells exhibit varying expression of epithelial and mesenchymal markers depending on the state of tumor differentiation. This offers a possibility for monitoring disease progression using minimally invasive procedures. EVs also offer the benefit of detecting molecular cargo of tumor origin and add the potential to detect circulating vesicle markers from tumors that lack invasive properties. This review integrates recent genetic insights of PC progression with developments in digital

pathology and early detection of tumor-derived circulating material.

Key words: Circulating tumor cells; Digital pathology; Early detection; Exosomes; Pancreatic cancer

Core tip: Pancreatic cancer (PC) is a leading cause of cancer-related death. PC mutations accumulate 20 years before patient death with metastatic mutations occurring late in the process. Metastatic risk increases dramatically when tumor diameter is greater than 1 cm. Most PC cases are diagnosed at late metastatic stages when survival is short. Outcomes could be improved if non-invasive methods could detect early stages of the disease and guide treatment decisions. Recent studies indicate this may be possible with application of digital pathology imaging, screening of CA 19-9 with additional markers, and detecting circulating tumor material in early-stage PC patients.

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INTRODUCTION

Pancreatic cancer (PC) is the third leading cause of cancer-related death in men and women in the United States surpassing breast cancer[1,2]. Projections indicate PC will outpace colorectal cancer and become the second leading cause of cancer-related death in the United States by 2020[2]. The majority of pancreatic tumors (90%) are classified as adenocarcinomas arising from the ductal epithelium with an annual incidence of 45220 patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) in the United States[1,3]. Estimates suggest that only 1.3%-10% of patients diagnosed with PC have familial basis for the disease where a genetic component is inherited from a relative[4]. The remaining majority of PDAC cases display large genomic heterogeneity[5]. Five-year survival is about 25% for localized stages but only 2% for advanced disease[1]. The best curative treatment is surgical resection, if performed early it presents a 5-year survival in 25%-30% lymph node negative patients but only 10% for those with positive lymph nodes[6-8]. Less than 20% of PC cases are diagnosed early enough for surgical intervention[2]. Relapse rate after surgery is typically high (80%) for this type of cancer and surgery is often followed by adjuvant chemotherapy or chemoradiation[2,9]. Approximately 80% of PDAC patients are diagnosed late when the disease becomes locally advanced or metastatic, where palliative chemotherapy is the only treatment option[10]. Since 1997, Gemcitabine has been commonly used over 5-fluorouracil (5-FU) albeit with only a modest median overall survival (OS) advantage of 5.6 mo (Gemcitabine) *vs* 4.4 mo (5-FU) in patients presented with advanced stage[11]. Extensive efforts have been made over the past decade, including numerous randomized phase Ⅲ clinical trials, to evaluate combinatorial drug treatments for patients with advanced disease[12]. To date erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, plus gemcitabine is the only course with a targeted therapy agent approved by the United States Food and Drug Administration (FDA) for first-line use in advanced PC[13-15]. FOLinic acid, Fluorouracil, IRINotecan, and OXaliplatin (FOLFIRINOX) and nab-paclitaxel/gemcitabine have emerged as combinatorial treatments with results that may reach the one-year survival barrier[16,17]. Adjuvant combination chemotherapy comprising gemcitabine with capecitabine has also shown statistically improved survival over gemcitabine monotherapy in PDAC subjects (ESPAC-4, Phase 3)[18]. Great focus has been extended into developing methods for improving early detection of the disease and exploring alternate treatment options that can extend survival in patients with late stage presentation[14]. This review provides description of the genetic fingerprints that drive disease progression and discusses selected features relevant to detection and treatment in this biological context. We further highlight recent advances in digital pathology, improvements in CA 19-9 testing, and detection of circulating tumor cells and tumor-derived extracellular vesicles (EVs) in biofluids of PC subjects (Table 1). Particular attention is made to literature that provides examples of material isolated from human PC subjects along with cell culture or animal model systems that explore mechanistic underpinnings.

PC PROGRESSION AND GENETICS

Computational modeling of primary pancreatic tumors supports the observations that metastatic probability increases exponentially with tumor size[19,20]. A patient with a primary tumor size of 1 cm in diameter is predicted to have a 28% probability of harboring metastasis at the time of diagnosis. This dramatically increases to 73% probability with a tumor size of 2 cm and elevates to 94% chance for a tumor size of 3 cm[20]. This clearly suggests that systemic treatments that target rapidly growing cells need to be administered early before log-phase growth is reached. For conventional therapies to improve survival, it will become paramount to detect early lesions before significant invasion takes course. The term pancreatic intraepithelial neoplasia (PanIN) was first coined in 1999 to describe ductal lesions which form as precursors to invasive cancer[21]. A progression model was soon after proposed where *HER-2/neu* overexpression and *KRAS* mutations are observed early, *p16 (CDKN2A/INK4a)* gene inactivation occurs at intermediate stages, with inactivation of *p53*, *DPC4*, and *BRCA2* occurring late[21].

Table 1 Summary of demonstrated clinical uses for digital pathology, circulating tumor cells and extracellular vesicles for pancreatic cancer

	Digital pathology	CTCs	EVs
Screening in population	Relies on invasive biopsies	Detection of KRAS mutations[92]	Early detection possibility (GPC1+ EVs)[117]
Diagnosis	Differential diagnosis of mucinous cancers[62]	Pancreatic CTC detected by ISET[82] and CellSearch[81]	GPC1+ EVs detected in IPMNs[117] EVs express mutated KRAS and p53 in PDAC serum[123] EVs detected in pancreaticobiliary cancers[124]
Staging	Early stage detection in mice[60] Distinguish Grade Ⅰ / Ⅱ in humans[61]	(C-MET, CK20, CEA) + CTCs elevated in late stages[96]	miR-17-5p in serum exosomes correlates with stage[128]
Prognosis	Potential	CTC positivity has prognostic value in locally advanced pancreatic cancer[81] CK20 expression in CTC indicates shorter overall survival[94]	Potential
Monitor treatment	Potential	CTC levels decrease during 5-FU therapy[91]	Potential
Drug sensitivity/ pharmacokinetics	CT scans can predict drug transport[35]	CTC apoptosis can be detected after 5-FU therapy[91]	Demonstrated for breast cancer[111]
Monitor recurrence	Potential	CTC positivity correlates with postoperative staging[94-97]	potential

EVs: Extracellular vesicles; CTCs: Circulating tumor cells; 5-FU: 5-Fluorouracil; PDAC: Pancreatic ductal adenocarcinoma; CT: Computed tomography; PDAC: Pancreatic ductal adenocarcinoma; CEA: Carcino-embryonic antigen.

This model predicts that PC evolves slowly with defined mutational characteristics and presents clinically at late stage. This progression paradigm of gradual pace has recently been challenged by Notta *et al*[22] who propose a punctuated equilibrium hypothesis where tumorigenic mutations arise from a cataclysmic event that rapidly leads to invasive cancer and metastasis. Data from this model suggests PC development is neither gradual nor follows the accepted mutation order which may be supported by observations showing that not all clonally expanded precursor lesions lead to a tumor lineage[5,22,23].

Recent evidence suggests that the development of metastatic cancers from primary tumors can take up to two decades, based on genomic sequence comparisons and mathematical analysis. The development of parental clones from an initiated tumor cell is estimated to take an average of 11.7 years, with an additional 6.8 years for expansion of metastatic subclones, and another 2-3 years before tumors disseminate to distant organs leading to patient death[24]. The founder mutations present only in the parental clones accumulate in a large number of driver genes involved in tumorigenesis such as *KRAS, TP53* and *SMAD4*. The resulting subclones, giving rise to metastatic lesions, contain additional progressor mutations which vary highly among subclones[24]. This suggests that distant metastasis occurs late during the genetic evolution of PC also supporting the punctuated equilibrium model of progression. These observations are consistent with findings that show more than 50% of the genomic rearrangements occur early during tumor progression being present in both primary and metastatic clones in the patient[25]. If these rearrangements could be narrowed to distinct genes or protein signaling pathways, they could serve as powerful targets for therapeutics made highly effective by reaching both primary and metastatic sites. In addition to identifying mutation hot-spots in metastatic clones, it will be important to compare founder mutations in primary tumors between patients with different survival outcomes to discover early factors that commit patients to a high risk course[26].

PCs were shown to have gene expression alterations in 69 gene sets, half of which cover at least twelve core signaling pathways with functional relevance in 67%-100% of observed neoplasias[27]. Even though these 12 overlapping cascades appear to be genetically altered in majority of the tumors, alterations of the pathway components themselves vary greatly between individual tumors[27,28]. This implies that therapies directed against these actionable targets may need to implement multi-targeted approaches based on selected patient subgroups, or consist of cocktails that effectively abolish entire signaling cascades[14,29].

A recent study performing whole-genome sequencing and copy number variation (CNV) analysis found a total of 857971 point mutations, insertions and deletions in 100 samples of PDAC[30]. The four most commonly mutated genes observed in PDAC patients are the oncogene *KRAS* (75%-90%), tumor suppressor genes *TP53* (74%), *CDKN2A/p16* (35%), and *SMAD4* (31%), along with inactivating mutations in the Rac exchange factor *PREX2*, the tumor suppressor *RNF43*, and the histone demethylase *KDM6A* observed in 10%-18% of subjects[30]. Focal amplification of druggable oncogenes such as *ERBB2, MET, FGFR1, CDK6*, *PIK3R3* and *PIK3CA* is observed at very low prevalence among only 1%-2% of patients[30]. Levels of protein expression or activity were not determined in these studies, however, to understand the functional significance of the focal amplifications. Integrated genomic analysis of PDAC identified 32 mutated genes that comprise 10 signaling

pathways: KRAS, transforming growth factor (TGF)-beta, WNT, NOTCH, ROBO/SLIT signaling, G1/S transition, SWI-SNF, chromatin modification, DNA repair and RNA processing[31]. Four tumor subtypes were identified based on differential expression of transcription factors and downstream targets: Squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine (ADEX) tumors. These tumor subtypes were sorted by gene programs to identify genetic factors that impact OS in PDAC subjects[31].

PDAC primary tumors can also be sorted into three distinct subtypes based on gene expression patterns and drug sensitivity: Classic, quasimesenchymal and exocrine-like[32]. The classic subtype, more sensitive to the EGFR inhibitor erlotinib, expresses high levels of adhesion-associated epithelial genes such as *AGR2, S100PBP* and *GATA6*. The quasi-mesenchymal subtype is more sensitive to gemcitabine and expresses high levels of mesenchymal genes such as *TWIST1* and *S100A2*. The exocrine-like subtype has high expression of tumor cell derived digestive enzyme genes such as *REG3A* and *PRSS1*[32]. These findings open the possibility for stratifying patients based on tumor gene expression patterns as a means for predicting drug sensitivity.

Taken together, these observations demonstrate that primary and metastatic tumors of the pancreas are highly heterogeneous and contain several distinct clonal populations with unique molecular signatures which develop over a long period of time. This makes targeted therapy difficult, unless common pathways are found that can be effectively blocked by personalized drug regimens[5].

PANCREATIC STROMA

Another source of genetic diversity can be found within the pancreatic stroma. PDAC cells are surrounded by a rich stroma that is typically far more abundant in cell types other than the tumor. Pancreatic stroma contains a variety of cells including stellate cells, immune cells, fibroblasts, vascular endothelial cells and the extracellular matrix which make up the tumor micro-environment (TME)[33]. TME plays a pivotal role in tumor behavior including proliferation, drug resistance, invasion and localized immune response[33,34]. A clinical study investigating intraoperative gemcitabine infusions during PDAC resection showed that high stromal density inhibits hENT1-mediated drug incorporation into the tumor[35,36]. Investigators in this study derived mass transport parameter (MTP) cutoff values based on expression of the nucleoside transporter hENT1 in the tumor, and pancreatic stromal density scores calculated from CT scans[35]. Applying MTP cutoffs to a cohort of 110 patients, who received gemcitabine therapy, revealed a 5-year survival rate of 40% in subjects with favorable transport parameters compared to a 15% survival rate in subjects who did not reach the parameter cutoff point[35]. This study demonstrates that stromal density and drug transport properties can be measured during surgery, using routine contrast-enhanced CT scans and immunohistochemistry, as a highly effective means for predicting significant response to cancer therapy. hENT1 expression in tumor cells permits bidirectional transport of pyrimidine nucleosides such as gemcitabine, capecitabine and 5-FU[37]. High expression of hENT1 in PC patients treated with gemcitabine is predictive of improved survival[36,38,39]. These studies open the possibility for determining drug sensitivity in resected patients through screening morphological features of the stroma combined with assessment of pharmacogenomic profiles[40].

The pancreatic stroma is enriched with large diversity of constituents, making it difficult to score clinically. A recent study applied a blind source separation technique called non-negative matrix factorization (NMF) to analyze gene expression from a microarray dataset that included 145 primary and 61 metastatic PDAC tumors in comparison to 134 normal tissue samples[41]. This technique effectively generated gene expression signatures sorted by tumor, stromal and normal cellularity. Patients that were identified with a "classical" tumor subtype had a median survival of 19 mo compared to patients with a "basal-like" tumor subtype that demonstrated a significantly worse survival of 11 mo. Additionally, two stromal subtypes were identified in patients: A "normal" subtype with 24 mo-median survival and an "activated" stromal subtype with significantly worse median survival of 15 mo. These techniques lead the way for identifying genetic markers that may otherwise be obscured by confounding material from normal and stromal tissue[41].

Mounting evidence supports the hypothesis that pancreatic TME s play a significant role in pathological outcome and treatment response and should therefore be clinically evaluated as a standard practice. The use of digital imaging combined with pharmacogenomic analysis could extend the application of existing treatments for personalized medicine. Best clinical outcomes come from early diagnosis of the disease. Leveraging the biological properties of pancreatic adenocarcinomas and their surrounding micro-environment for early detection and diagnosis would provide maximum benefit for patient survival.

CURRENT DIAGNOSTIC METHODS USING SERUM

Presently, there are no suitable PC screening strategies effective for early detection of PC in the general population. Diagnosis of PDAC is made by pathological assessment of a tissue biopsy. The current gold standard is *via* an endoscopic ultrasound technique coupled with fine needle aspirations (EUS-FNA) which has a sensitivity of 75%-94% and specificity of 78%-95%[8,42]. For patients who have non-diagnostic FNAs or cannot undergo endoscopy, treatment decisions are based on imaging or determining CA 19-9 serum levels[8]. The only serum biomarker approved by the FDA for PC is the sialylated Lewis (a) blood group antigen CA 19-9 which is not tumor specific and is frequently elevated during many malignancies,

Table 2 Clinical uses for biomarker panels that increase predictive value of CA 19-9 for pancreatic cancer

	CA 19-9	Sensitivity	Specificity	Ref.
Screening in population	EUS-FNA	75%-94%	78%-95%	[42]
	CA 19-9[1]	60%-70%	70%-85%	[45,46]
Differential diagnosis	CA 19-9	60%	83%	[44]
	CA 19-9 + CA 125	87%	77%	[44]
	CA 19-9 + ICAM-1 + OPG	78%	94%	[49]
	CA 19-9 + CEA + TIMP-1	71%	89%	[49]
Staging	PAM4-reactive mucins	76%	85%	[51]
	CA 19-9 + PAM4-reactive mucins	84%	82%	[51]
Monitor treatment	Response to chemotherapy			[47]
Monitor recurrence	Low levels post-surgery correlate with survival			[45]

[1]Values reflect subjects presented with pancreatobilliary disease. EUS-FNA: Endoscopic ultrasound and fine needle aspiration; OPG: Osteoprotegerin; ICAM-1: Intercellular adhesion molecule 1; CEA: Carcinoembryonic antigen; TIMP-1: Tissue inhibitor of metalloproteinases 1; clivatuzumab monoclonal antibody (PAM4) to MUC5AC.

pancreatitis, cholangitis, obstructive jaundice, hepatobiliary cancer, and benign biliary obstruction[43,44]. CA 19-9 alone has not been shown to be an effective screening marker for PDAC among the general population based on most studies[45]. However, sensitivity (60%-70%) and specificity (70%-85%) of CA 19-9 improve significantly in patient cohorts presented with pancreatobiliary disease[45,46]. Low serum CA 19-9 levels following surgery correlate with improved survival[45]. Oncologists occasionally use CA 19-9 to track response to chemotherapy but the predictive significance of CA 19-9 for this purpose has reported some variability[43,45,47].

Measuring CA 19-9 in combination with other markers such as CEA, CA242, and TIMP1, however, was shown to improve its predictive value (Table 2)[45,48,49]. Barnett *et al*[49] could identify PDAC patients using two independent panels: CA 19-9, CEA, and TIMP-1; and a second panel containing CA 19-9, ICAM-1, and OPG. Both panels demonstrated increased sensitivity and specificity over CA 19-9 alone (Table 2)[49]. Recently, O'Brien *et al*[44] discovered CA 19-9 (> 37 U/mL) and CA 125 (> 30 U/mL) serum levels can be elevated up to two years before PDAC diagnosis based on a nested case control study. CA 125 has been reported to distinguish malignant from benign PC tumors with 60.8% sensitivity and 83.3% specificity which improved to 87.8% and 77.8% respectively when combined with CA 19-9[50]. PAM4, an antibody which binds mucin MUC1 and MUC5AC epitopes expressed in PC, was capable of identifying 64% of stage Ⅰ PDAC patients with high discriminatory power compared to those with benign pancreatic disease[51]. PAM4 is capable of distinguishing normal pancreas from PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 lesions, intraductal papillary mucinous neoplasia (IPMN) lesions, as well pancreatic adenocarcinomas of various grades[52]. Combining CA 19-9 with a PAM4-reactive marker improved sensitivity (84%) without a loss in specificity (82%) in a serum-based enzyme immunoassay (EIS)[51]. Despite some propensities for false positivity, CA 19-9 continues to be a benchmark serum marker for evaluating PC in the clinical setting. It will be important to test combinations of other markers in addition to CA 19-9 to improve its diagnostic utility in larger populations.

TUMOR IMAGING AND DIGITAL PATHOLOGY

In addition to biopsies and serum marker tests, lesions and primary tumors can be characterized by clinical imaging. Computed tomography (CT) and magnetic resonance imaging (MRI) are the most frequently used imaging method for diagnosis and clinical staging[33,43]. Additional screening approaches using imaging multi-modalities include endoscopic ultrasonography (EUS), magnetic resonance cholangiopancreatography (MRCP), and endoscopic retrograde cholangiopancreatography (ERCP)[4]. However, these approaches are limited to surveillance centers with robust PC programs and are typically only performed on high-risk patients[53,54]. MRI and EUS have been proposed for use as first line modalities but often fail to distinguish benign from malignant lesions[55]. Emerging imaging modalities and molecularly targeted imaging agents are of great interest as early detection strategies but may be cost prohibitive and inaccessible to many patients[56].

Upon diagnosis, patients are staged based on the AJCC 7th Edition Staging Manual criteria before proceeding to surgery[8,57]. This is typically accomplished through cross-sectional imaging (CT or MRI) along with tissue biopsy[8]. Among those staged with resectable disease by biopsy, only 70%-85% actually present with resectable tumors, intraoperatively[8]. This indicates a need for improvements in staging methodology which may be enhanced by digital pathology[8]. The field of digital pathology has recently grown to complement histological diagnosis performed by pathologists[58]. These methods extract and quantify histological features from whole slide images thus improving on the subjective nature of the work[59].

Langer *et al*[60] developed a method that can accurately predict early pancreatic lesions with a 93% success rate

in an independent test set using tissue obtained from mouse models of early-stage PDAC. The program uses a top-down object learning paradigm similar to the methodology used by human pathologists. Initially, ducts, nuclei and tumor stroma are identified and segmented. From those, secondary morphological features such as duct deformation and nuclei malformations are measured. These data sets are then used to train a predictive model that distinguishes normal tissue from premalignant cancer lesions[60]. Similar techniques can be extended to accomplish classification of PDAC by grade using human tissue samples[61]. Diagnosis of PDAC was made based on three parts: Segmentation and feature extraction; model learning and validation; and diagnosis. Training data measuring ducts, consisting of the lumen and epithelial nuclei, can distinguish normal human subjects and those with grade Ⅰ and grade Ⅱ PDAC with an accuracy of 94%[61]. Automated systems have been developed for making a differential diagnosis of rare lesions such as cystic neoplasms of the pancreas using human biopsy tissue[62]. Song *et al*[62] were able to distinguish benign serous from malignant mucinous cystadenomas using a computer-aided design technique. Cystic regions were identified and epithelial cells surrounding the lumen were discerned. Three classes of features were analyzed by the program to achieve a differential diagnosis: The number and size of cysts, characteristics of the surrounding epithelium, and indication of mucus production[62].

Current applications of digital pathology for PC do not offer much more beyond histological diagnosis performed by a pathologist but indicate potential for detecting early lesions. Improvements could be made, for example, by developing digital pathology methods for images annotated with clinical data from population-based repositories. This could potentially aid the discovery of morphological features associated with treatment and survival outcomes.

The intended goal beyond research is to incorporate digital tools into clinical practice as a way to standardize histological diagnosis in patients at high risk of developing the disease. This could improve staging and determination of resectability. Some concerns raised include public health consequences if misdiagnosis is caused by improper use or analysis of poor quality images[63]. The Food and Drug Administration recently released a guidance for technical performance assessment of digital pathology whole slide imaging (WSI) devices[64]. Currently, WSI devices are classified as Class Ⅱ for methods that provide adjunct analysis after a primary diagnosis is made using glass slides. WSI devices which make a primary diagnosis alone are classified as high-risk Class Ⅲ devices if their intended use is new and lacks a Class Ⅱ predicate. A *de novo* process provides a less resource-intensive approval path to Class Ⅰ/Ⅱ classification if special controls are presented that provide reasonable assurance of safety and effectiveness. A more clearly-defined approval process for manufactures would enhance innovation and commercialization potential of digital pathology instruments and software[65].

DETECTING TUMOR CELLS IN CIRCULATION

Performing invasive biopsies for routine screening of the general population is not reasonably a feasible option. Detecting tumor material in the blood or other biofluids would be ideal for many reasons. A test assessing a panel of markers in biofluids could be ordered by physicians in most clinical centers, and collected by non-invasive or minimally invasive procedures. Performing additional tests using the same starting material could easily lead to diagnostic refinement. Diagnostic tests can be expanded to cover non-tumor biomaterial such as components of the immune system, blood/serum, pancreatic juice, stool, oral and gut microbiota, and markers of metabolic activity. Given patient variability, measuring systemic profiles of markers not directly derived from tumors may not yield the specificity and sensitivity necessary to accurately determine risk for developing advanced PC. A search for "pancreatic cancer" in the published literature can easily generate over 50000 returns which documented more than 2500 individual genes as potential PC biomarkers due to their overexpression patterns[66]. A compendium of PC biomarkers identified at least 1000 molecules with evidence of upregulation in precursor lesions[66]. Early detection of these precursor lesions particularly before invasive cells establish colonization would be ideal.

A critical study, using genetically engineered mouse models of PanIN, showed that cells from preneoplastic lesions can breach the basement membrane and spread into the stroma[67]. Contrary to conventional wisdom, these cells undergo epithelial-to-mesenchymal transition (EMT) and enter the blood into circulation with no evidence of carcinoma. These findings suggest that EMT transition can occur as an early phenomenon even before histologic emergence of cancer. These cells acquire a mesenchymal phenotype, exhibit stem cell properties, have tumor-initiating capacity, and are most abundantly observed at inflammatory foci[67]. Induction of pancreatitis and immunosuppressive treatment with dexamethasone have strong effects on dissemination supporting a link between early precursor cell invasion and localized inflammation[67]. Typical circulating tumor cell (CTC) markers such as EpCAM are expressed in less than 20% of the PanINs in this model system[67]. This has implications for commercially available methods which may overlook these circulating precursors, because they rely on such epithelial markers for CTC detection.

EMT in primary cells was shown to be associated with acquisition of stem cell-like characteristics[68]. Both normal and cancer stem cells possess the ability to self-renew and produce differentiated progeny[29]. Cancer stem cells are further functionally defined by having enhanced tumor initiating capacity when transplanted to a permissive host[67]. *In vivo*, CTCs detach from the primary tumor and enter the blood where they can be

transported to distant sites with only 0.01% surviving to form metastases[69]. CTC detection has been extensively used for prognosis (progression free survival and OS) and predicting response to treatment in breast, prostate, colorectal and lung cancers[70-74]. CTCs have also been detected in PC patient samples but their prognostic potential remains to be optimized outside the limitations of a small sample of subjects[69,75-77].

CTC DETECTION METHODS

Circulating tumor cells are present at very low concentrations in the blood, typically one CTC per billion blood cells. For this reason, CTCs need to be enriched to differentiate them from the vast hematopoietic cell background, and characterized to verify their tumor origin[69]. Several enrichment media for density-gradient centrifugation are commercially available including LymphoPrep™ (Axis-Shield), Ficoll-HyPaque™ (Sigma-Aldrich), Oncoquick® (Greiner Bio-One), and RosetteSep ™ Human Circulating Epithelial Tumor Cell Cocktail with SepMate™ (StemCell Technologies). Enrichment is typically followed by targeted isolation. Four strategies are available to isolate and capture CTCs: Positive selection using antibodies attached to solid-support, negative selection, cell size-based methods such as filtration, and physical property-based methods[77]. Most CTC detection methods rely on either positive immunoselection of cells expressing the epithelial cell adhesion molecule (EpCAM) or negative selection by depleting leukocytes from the blood using CD45-binding antibodies[78]. Commercial immunomagnetic bead separation systems are available including EasySep cell separation (StemCell Technologies), Dynabeads (Invitrogen), CellSearch CTC system (Janssen Diagnostics) and MACS (Miltenyi). CellSearch CTC is the only system approved by the FDA for capturing and enumerating CTCs of epithelial origin by $CD45^-$, $EpCAM^+$ and $cytokeratin^+$ selection[79]. CellSearch has been cleared by the FDA for management of breast, colorectal and prostate cancers and has also been tested in PDAC patients with detection rates varying from 11%-45%[44,78,80-85].

Once isolated, circulating tumor cells are typically characterized by immunocytochemical (ICC) staining or nested real-time polymerase chain reaction (RT-PCR). Detection strategies typically assess epithelial mRNA profiles which include *EpCAM*, epithelial carcinoembryonic antigen (*CEA*), *CEACAM5*, *CK19*, *BIRC5* and *MUC1*[76]. There are currently more than 40 assay platforms for CTC detection and enrichment that have been widely publicized[86]. Among these, the utilization of microfluidics and microarray technology in CTC detection is expanding.

CTC detection was investigated as a prognostic tool in a LAP07 international multicenter randomized study to assess if patients with locally advanced pancreatic carcinoma (LAPC) would benefit from chemoradiotherapy over continuation of chemotherapy[81]. Bidard *et al*[81] were able to achieve a CTC detection rate of 11% using a low cut-off of one or more CTCs/7.5 mL of blood using the CellSearch system. This is lower than the 50% detection rate typically reported for metastatic PC patients[84]. CTC positivity nonetheless was a prognostic factor for OS which was lower in CTC positive LAPC patients[81]. More CTCs can be detected in the blood of PC patients using ISET (Isolation by Size of Tumor Cell) based on a comparative study which found detection of 26 CTCs/7.5 mL blood using ISET compared to 2 CTCs/7.5 mL blood by CellSearch[82]. ISET also detected CTCs in a much higher proportion of patients (93%) *vs* CellSearch (40%)[82]. ISET is a filtration-based, marker-independent method that sorts by cell size and morphology using filter modules offered by a company started by the inventor of the technology (Rarecells Diagnostics)[87,88]. Thus ISET may offer a significant advantage over CellSearch which relies on expression of EpCAM for CTC identification. PDAC cells, among carcinomas, are more prone to epithelial-mesenchymal transition (EMT) which reduces the expression of EpCAM[78,89,90]. This presents a problem for PC detection using CTCs as most of the current CTC detection methods rely on EpCAM or other epithelial molecules for CTC detection[69,86].

CTC CHARACTERIZATION

There exists a critical need for the development of assays that can additionally identify CTCs which undergo EMT and lose expression of typical epithelial surface antigens. Ren *et al*[91] detected CTCs in peripheral blood of advanced stage PC patients before (in 80% of patients) and after treatment (in 29% of patients) with 5-FU by immunostaining for CA19-9 and CK8/18 expression. The mean concentration of blood CTC decreased from 16.8 cells/7.5 mL of blood before chemotherapy to 3.8 cells/7.5 mL blood after a seven-day cycle of 5-FU chemotherapy[91]. Evidence of apoptosis induced by 5-FU was observed in CTCs obtained from patients and in pancreatic cell line models (PL45 and PANC-1 cells)[91]. These studies open the possibility for using CTC assays to monitor chemotherapy efficacy and extent of remission although they fail to selectively identify mesenchymal antigens expressed by CTCs. Other potential mesenchymal protein marker candidates include Cadherin 2, Vimentin, Snail/Slug, zinc finger E-box binding homeobox1 (ZEB1), and Twist family basic helix-loop-helix transcription factor 1 (TWIST1)[79]. These mesenchymal markers could be combined with PC-specific markers to increase specificity.

To verify tumor origin, isolated CTCs can also be screened for genes expressed or mutated predominantly in PC such as *KRAS*. Court *et al*[92] detected *KRAS* mutations in 92% of PC patients using a NanoVelcro/laser capture microdissection (LCM) platform. This technique captures CTCs on a microfluidic chip using biotinylated anti-EpCAM antibodies and is followed by identification through ICC staining of CD45, CEA, and staining of pancytokeratin for nuclear morphology. Mutations in *KRAS* were not observed in white blood cells and overall reliability of the assay required isolation of only 10-100 circulating tumor cells[92]. Chausovsky *et al*[93] detected the

expression of Cytokeratin 20 (*CK20*) in 22/28 PC patients using RT-PCR analysis of peripheral blood CTCs. Soeth *et al*[94] found that *CK20* was expressed in CTCs of 33% of patients in a larger cohort (n = 154) who had significantly shorter OS. Cytokeratin 7 (*CK7*) and cytokeratin 20 (*CK20*) are expressed in a variety of epithelial neoplasms including majority of pancreatic carcinomas (62%)[95]. A variety of commercial platforms are now available for detection of amplified CTC DNA such as TruSeq Amplicon (Illumina) and Ion Torrent AmpliSeq™ (Life Technologies).

Levels of RNA expression can also be measured by RT-PCR or directly imaged by *in situ* RNA hybridization using platforms such as ViewRNA™ CTC Platform (Affymetrix). Zhou *et al*[96] measured mRNA expression of *h-TERT, C-MET, CK20*, and *CEA* by RT-PCR in CTCs isolated by immuno-magnetic enrichment using EpCAM. This method can distinguish PC patients from benign control subjects with high degree of specificity. Further, when pancreatic patients were in later stages, the expression rate for C-MET (67%), CK20 (75%) and CEA (75%) were statistically higher than during earlier stages[96]. These findings open the utility of CTC detection for monitoring disease progression. Two independent studies have also found that preoperative CTC positivity correlated with postoperative staging[94,97]. This indicates that in addition to diagnostic value, CTC detection has prospects for PC staging[8].

The genetic content of CTCs can also be sequenced for molecular discovery[92]. Yu *et al*[98] adapted a microfluidic device to capture CTCs which were subjected to single-molecule RNA sequencing. Using a mouse PC model, *Wnt2a* gene was identified to be enriched in CTCs isolated from mice and in 5/11 human PC cases[98]. Ting *et al*[99] used focusing-enhanced microfluidic capture of CTCs (CTC-iChip) from primary PC tumors followed by deep-RNA sequencing. RNA-seq profiles identified enrichment of stem-cell-associated genes such as *Aldh1a2* and the extracellular growth factor binding protein *Igfbp5* which localized focally at the tumor epithelial-stromal interface[99]. CTCs of mouse and human origin also expressed elevated levels of gene expression of the stromal-derived extracellular matrix protein (SPARC), which increases invasive and migratory potential of PDAC cell lines[99]. Whole exome sequencing of CTCs has been successfully accomplished in metastatic prostate cancer cells, PDACs, and pancreatic carcinoma neoplasms with acinar differentiation[100-102].

To improve prognostic value, CTC analysis can be combined with other methods such as direct detection of circulating free DNA (cfDNA) in the blood. Mutated *KRAS* cfDNA, isolated from plasma, was observed in 26% of patients with resectable and advanced stage disease and correlated strongly with decreased OS compared to mutant *KRAS* free subjects (60 d *vs* 772 d)[85]. Patients with panreatobiliary carcinomas were accurately diagnosed using cfDNA sequencing with a 92% sensitivity and 100% specificity[103]. CTCs were detected in peripheral blood of 20% of metastatic disease patients using the CellSearch system by CD45 positive cell depletion. CTC positive PDAC patients had decreased OS of 88 d (95%CI: 27-206) compared to 393 d (95%CI: 284-501) in CTC negative subjects[85]. Circulating tumor DNA (ctDNA) can also serve as a detection strategy on its own or in combination and can be found in other articles that focus on this topic[78,104,105]. For example, Berger *et al*[104] were able to distinguish patients with Intraductal Papillary Mucinous Neoplasm (IPMN) lesions from controls by detecting mutation hot-spots in circulating cell-free DNA from patient blood samples.

Collectively, the utility of circulating tumor cells as a diagnostic marker in PC is gaining more ground. CTC detection offers the benefit of a low-risk safety profile which may be a cheaper alternative to FNA biopsies[8]. The cost of obtaining a diagnosis by EUS-FNA in the United States can be approximately $16000 compared to $370 Medicare reimbursement for the CellSearch CTC-based Assay[106,107]. A broader range of epithelial and mesenchymal markers are needed to create techniques that adequately capture a wide range of PC-specific circulating tumor cells. Finally, selected CTC techniques need to be tested in larger patient cohorts to pass the same FDA clinical guidelines that made the CellSearch CTC-based assay a successful clinical tool for breast, prostate and colon cancer.

EVS

The study of EVs has gained significant momentum in recent years, because their cargo represents material of tumor which can shed light on the state of disease progression. EVs are membrane-bound organelles secreted by a variety of cells including cancer cells. The cytosol-derived lumen of EVs is enclosed by a lipid-bilayer forming a delivery vehicle for a variety of nucleic acids, proteins and lipids which can be horizontally transferred into recipient cells altering their biological properties. This allows cancer cells to continually modify their local microenvironment as well as distant sites when EVs enter circulation[108]. Because the molecular composition and function of these organelles represents their tumor origin, insight into EV biology provides great potential for tumor screening, diagnosis and prognosis. However, not all EVs are alike. The subcellular origin determines the type of cargo and mechanism of release from the cell. Large microvesicles (100-1000 nm) that bud outward from the plasma membrane are called ectosomes or ARRDC1-mediated microvesicles (ARMMs)[109]. Small EVs (30-150 nm) are called exosomes, which originate as intraluminal vesicles found in endosomal membranes and are secreted through fusion of multivesicular bodies (MVB) with the plasma membrane[110]. Several studies have identified exosomal subtypes based on molecular content that may hold diagnostic and prognostic value for diseases such as PDAC[110,111].

Exosomes play an active role in disease progression by promoting tumorigenesis, metastasis, tissue remodeling, immune evasion, and chemoresistance[111,112]. This is reported to be achieved by the delivery of microRNA,

mutated genomic DNA fragments, proteins and lipids which alter the biology of tissues that take up cancer-derived exosomes[112]. Exosomes offer several detection advantages over other biomarkers. Because exosomes travel across the endothelium into circulation they can be detected in serum and/or urine which can be collected over time when monitoring a patient[112]. Exosomal content can be dispersed within the lipid membrane bi-layer but can also be found in the lumen where it is protected from degradation by external nucleases and proteases[113]. Once exosomes are isolated, their content can be much easier to detect by sensitive techniques such as RT-PCR, next generation sequencing, gene expression microarrays, and mass spectrometry[111,114]. The first challenge in establishing exosome biomarkers as clinical tools depends on the ability to isolate them in sufficient quantity at high purity.

Initial isolation depends on crude physicochemical properties such as particle size, density and solubility. Isolation by differential centrifugation is the most classical method used by the biomedical research community. However, differential centrifugation typically results in low yield and always presents with some degree of contamination[108]. Recent developments have improved yield and purity through precipitation, affinity-based sorting by magnetic beads, and particle size-based isolation such as ultrafiltration and size exclusion chromatography[108,113]. Exosome isolation kits are now readily commercially available[108,115]. The identity and enrichment of exosomes in a biochemical fraction can be further defined by detection of endosome-specific tetraspanins (CD9, CD63, CD81), membrane transport and fusion proteins (flotilin, GTPases), MVB biogenesis-related proteins (Syntenin, Alix, ESCRT, TSG101), and heat-shock proteins (Hsp60, Hsp70, Hsp90)[110,113,116].

EXOSOMAL CARGO

Given that most cells secrete exosomes, it can be a difficult task to distinguish cancer-specific material to that of healthy cells. When evaluating pathological relevance of exosome studies, purification methodology, exosome identification and presence of cancer-specific markers are essential components that should be taken into consideration. One of the most widely acclaimed PDAC exosome studies was recently presented by Melo *et al*[117]. The authors identified the presence of heparin proteoglycan GPC1, in exosomes isolated from breast and PC patients by ultracentrifugation and sucrose density gradient separation (followed by CD9, CD81 and flotillin 1 detection). Baseline GPC1 positivity was found in only 2.3% of healthy donors while elevated GPC1 expression was found in 75% of breast cancer subjects and among 100% of pancreas cancer patients (n = 190). Relative concentrations of exosomes were much higher in the sera of cancer patients compared to healthy subjects. $GPC1^+$ exosomes were also detected prior to formation of PanIN lesions in 16-d-old mouse models of PDAC ($Ptf1a^{cre/+}$; $LSL\text{-}Kras^{G12D/+}$; $Tgfbr2^{L/L}$) with increased proportionality over time. Serum $GPC1^+$ exosomes in these models were present in circulation early before the onset of histological signs or MRI-detectable lesions. Further, the authors were able to use GPC1 positivity to distinguish healthy donors and those with benign pancreatic disease (BPD) from patients with histologically validated PC precursor legions (intraductal papillary mucinous neoplasm-IPMN) with a high degree of specificity (75%) and sensitivity (82%). Taken together, these findings suggest that PC cells secrete elevated levels of GPC1 positive exosomes which may be useful for early detection of tumors even prior to histologic manifestation[117].

EGFR is a receptor tyrosine kinase (RTK) activated in a subset of PC cells[118]. Adamczyk *et al*[119] found pancreatic cell lines (BxPC3, MiaPaca2, Panc1, Paca44 and A818-4 cells) secrete a 110 kDa soluble form of the EGFR ligand-binding extracellular domain (sEGFR) directly into conditioned media. A 170 kDa intact receptor and a 65 kDa processed form, including the intracellular kinase domain, are secreted as constituents of exosomes. Exosomes were separated from the secretome by ultracentrifugation and confirmed by exosome markers Alix, CD9, CD63 and Syntenin. The full-length EGFR was enriched 20-fold in exosomes along with 1600 other proteins found in the fraction by mass spectrometry[119]. The reason for compartmentalized release of these processed EGFR forms is currently not known. Soluble EGFR may provide a method for distant receptor transactivation or may confer EGFR positivity in cancer cells lacking EGFR expression. $EGFR^+$ exosomes may also enhance drug resistance by serving as a decoy for therapeutic antibodies. This has been observed in $HER2^+$ exosomes secreted by breast cancer cells that were shown to inhibit cell proliferation effects of Trastuzumab but not Lapatinib[120]. The next important step will be detecting $EGFR^+$ exosome in healthy and PC patients. Whether these isoforms possess any oncogenic mutations also needs to be explored before clinical use of these exosomes as cancer-specific biomarkers is considered.

KRAS is an oncogene that is mutated in 90% of PC cases[121,122]. Kahlert *et al*[123] isolated large (> 10 kb) double stranded genomic DNA fragments from EVs originating from PC cell lines and from serum of PDAC patients. Exosomes were purified from cell lines (Panc-1, T3M-4) and serum isolated from patients prior to surgical resection using ultracentrifugation after filtration. Exosomes were further verified by expression of CD9, TSG101, and CD63 by FACS analysis. By using whole genome sequencing, the authors demonstrated that PDAC serum exosomes contain not just mutated *KRAS* and *p53* oncogenes but also genomic DNA fragments spanning all chromosomes[123]. This suggests that genomic fragments can be isolated from purified PC exosomes and sequenced for analysis. Exosomes isolated from peripheral blood and pleural effusions can be sequenced to profile the genomes and transcriptomes of patients with pancreaticobiliary cancers[124]. Traditional tissue biopsies for these deeply located visceral cancers are difficult to safely acquire in

Table 3 Challenges and potential solutions for pancreatic cancer diagnosis and treatment

Challenges	Potential solutions
Metastatic probability increases dramatically with larger tumor size	Promote development of early detection methods (circulating tumor cells, extracellular vesicles, molecular cargo in CTCs and EVs, cfDNA, ctDNA)
Tumor mutations develop up to two decades with metastatic mutations occurring late in the process	Identify founder mutations that correlate with unusual survival outcomes
Pancreatic stroma influences treatment sensitivity	Promote research on stromal characterization
Transporter expression in the tumor impacts drug delivery	Identify expression features that correlate with treatment sensitivity to a variety of drugs
CA 19-9 is not pancreatic cancer specific	Promote development of assays for biomarker panels that increase CA 19-9 utility that will be eligible for FDA approval
Prediction of resectability is only 70%-85% accurate	Improve staging based on biopsies by implementing clinical use of digital pathology methods
No FDA-approved digital pathology methods exist for pancreatic cancer	Combine digital pathology with accepted primary diagnostic methods and test special controls for digital imaging that will permit FDA application through a more streamlined *de novo* pathway

CTC: Circulating tumor cells; EVs: Extracellular vesicles; cfDNA: Circulating free DNA; ctDNA: Circulating tumor DNA; FDA: Food and Drug Administration.

less specialized clinical centers. These studies create the possibility of performing genomic panel tests to identify oncogenic material from exosomes isolated from patients suspected of having elevated risk of PC or those where traditional biopsies are not feasible to obtain.

In addition to carrying genomic DNA, exosomes can also directly inhibit translation or target mRNA for degradation through the delivery of microRNA[125]. Exosomal miR-21, miR-212-3p and miR-203 and have been shown to enhance chronic pancreatitis, modulate immune response, and induce drug resistance[125-127]. Que *et al*[128] found elevated levels of miR-17-5p in serum exosomes of PC patients which correlate with metastatic stage, compared to healthy controls. Levels of exosomal miR-21 were also higher in PC patients *vs* healthy and chronic pancreatitis subjects but did not correlate with PC differentiation or stage[128]. The concentration of EVs in serum or plasma is almost one thousand times higher than in urine, a less invasive biofluid where exosomes remain stable at room temperatures for up to a week[115]. Ymir Genomics has developed a novel precipitation reagent, Ymirite, which isolates extracellular nucleic acids and vesicles from urine samples. Exiqon offers two exosome enrichment kits (miRCURY) for serum/plasma and urine isolation and a qPCR detection system (LNA™) for miRNA detection which enables profiling of biofluids where microRNA levels are extremely low. Further improvements in exosome and oncosome cargo characterization will significantly improve the clinical prospects of EVs.

EXOSOMAL MARKERS THAT INDICATE PATHOGENIC EFFECTS OF PDAC

Once released into circulation, the destination of tumor-secreted exosomes can be directed through expression of membrane proteins that guide cellular targeting such as integrins, tetraspanins, phosphatidylserine receptors and heparin sulfate proteoglycans[117,129-131]. These features enable exosomes to reach distant sites where they can exert pathogenic effects secondary to the primary cancer. For example, PDAC derived exosomes have been shown to promote liver metastasis through expression of macrophage migration inhibitory factor (MIF) which induces a fibrotic microenvironment when taken up by liver resident Kupffer cells[112,132]. PDAC derived exosomes can also secrete TGF-beta which activates hepatic stellate cells to secrete fibronectin which in turn arrests bone-marrow derived macrophages and neutrophils to produce pro-tumorigenic cytokines in the liver[127,132].

Diabetes is a risk factor for PC but the association is complex[133]. Studies by Javeed *et al*[134] suggest that adrenomedullin (AM), secreted into circulation by pancreatic exosomes, reaches remote pancreatic beta cells to induce beta-cell dysfunction by inhibiting insulin secretion. The authors showed AM^+ exosomes, isolated by differential centrifugation, are secreted into cultured media by PC patient-derived primary cell lines as well as into portal/peripheral venous blood of PC patients. Additionally, these AM^+ exosomes also contain CA 19-9 making them an attractive PC biomarker[134]. Another PDAC-exosomal protein Bip, also impairs insulin secretion through interactions with pro-insulin[127]. These studies hold promise for potential diagnostic methods which may predict secondary complications to PC.

Detection of exosomes and their cargo presents some attractive qualities as a liquid biopsy technique. Advantages include the ability to capture tumor-derived material circulating before and during metastatic colonization, enable monitoring of treatment effectiveness and recurrence, enhance prognostic capability based on classifying molecular signatures, and serving to indicate secondary complications. Vesicle enrichment methods have been streamlined and standardized through the availability of commercial kits. The discovery of highly cancer-specific exosomal markers such as GPC1 will provide a foundation that could serve as the basis for

accurate and non-invasive diagnostic tests.

CONCLUSION

The genetic evolution of PC is complex and may take up to two decades with metastatic mutations occurring relatively late in the process. Diagnosis is made at late stage where large genetic heterogeneity is observed within the tumor. With genotyping costs decreasing, it may be possible to predict drug sensitivity following resection through a combination of genomic profiling of the tumor, stromal density image processing and transporter expression determination. Digital analysis of the stroma from CT images has demonstrated the ability to predict a significant survival benefit for patients who undergo gemcitabine treatment. These methods pave the way for future applications in digital pathology as a means to increase prognostic potential and augment treatment decisions for personalized medicine.

While there is some room for refining existing treatment options to extend survival of late-stage PC patients, overcoming challenges for early detection of the disease will be paramount to significantly decrease the burden on the population (Table 3). Detecting physiologically relevant markers in exosomes and circulating tumor cells offers an advantage of testing with little or no discomfort to the patient. This creates the possibility to obtain serial samples of body fluids over time to allow monitoring of disease progression while eliminating risks associated with invasive biopsies. Combining information gained from these two types of tests could potentially increase diagnostic potential during early stages of PC development.

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This article is dedicated to Sgt. Mark Diehl.

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